

ROLE OF p24 PROTEINS IN REGULATING REPRODUCTIVE BEHAVIOR

A Senior Scholars Thesis

by

STEPHANIE THERESA GRADY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2010

Major: Biology

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Approved by:

Research Advisor:

Associate Dean for Undergraduate Research:

Ginger E. Carney

Robert C. Webb

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ABSTRACT

Role of p24 Proteins in Regulating Reproductive Behavior. (April 2010)

Stephanie Theresa Grady
Department of Biology
Texas A&M University

Research Advisor: Dr. Ginger E. Carney
Department of Biology

The organism *Drosophila melanogaster*, otherwise known as the fruit fly, has proven to be a respectable genetic model for analyzing behavior. Genes function within signaling pathways to regulate a variety of behavioral responses, such as ovulation and egg laying. Our gene family of interest consists of nine *p24* genes that encode for 24 kD transmembrane proteins. When the expression of *p24* genes *logjam*, *eclair*, or *baiser* is lost, adult females do not oviposit eggs. These genes may be responsible for trafficking cargo vesicles from the endoplasmic reticulum to the Golgi apparatus within cells. Therefore, we hypothesize that the function of p24 proteins is to traffic an ovulation or oviposition signal within a specialized set of cells. Unfortunately specificities regarding the function and localization of p24s remain unidentified.

In order to determine function, we characterized p24 localization in a variety of *D. melanogaster* tissues using p24-specific antisera. We discovered that p24 proteins are

expressed in an assortment of tissues in the fly, especially in the nervous system and reproductive tissues.

Co-immunostaining of p24 proteins and peptidergic cell markers showed an association between p24-expressing and peptide secreting cells, thus supporting the hypothesis that p24s function in the relaying of signals for neuropeptide secretion. If the trafficking of signals is blocked due to the lack of *p24* gene function, then neuropeptides controlling ovulation and egg laying would not be secreted, and eggs would not be oviposited as seen in some *p24*-deficient animals. There is evidence that two specific neurotransmitters, octopamine and glutamate, synergistically control egg laying behavior. It is possible that *p24* mutants are deficient in the release of these neurotransmitters. To test this hypothesis, *eclair* and *logjam* mutants were fed octopamine and glutamate in order to restore egg-laying behavior. We discovered that feeding mutant females these neurotransmitters did not restore egg laying. Therefore, the defect is most likely not due to the loss of neurotransmitters. However, we have theorized ideas of possible issues with the *p24*-deficient fly.

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I would like to thank my research advisor Dr. Ginger Carney. Her encouragement and knowledge have been extremely valuable over the past three years. She truly has an exceptional gift of molding college students into scientists.

I would also like to thank Lisa Ellis, Christoph Schwedes, Kara Boltz, and Sehresh Saleem for their continuous help and support in the lab.

NOMENCLATURE

Bai	Baiser
CS	<i>Canton-S</i> (wild-type strain)
DNA	Deoxyribonucleic Acid
Eca	Eclair
ER	Endoplasmic Reticulum
kD	kilo-Dalton
Loj	Logjam
mg	milligram
mL	milliliter
RNA	Ribonucleic Acid
RNAi	RNA interference
UAS	Upstream Activation Sequence

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CHAPTER I

INTRODUCTION

Mutations: Departing from the original blueprint

Many characteristics of behavior are controlled by genes— molecular blueprints encoded on chromosomes of an organism. Through signaling pathways, genes carry out the expression of multiple phenotypes, the visual representation of an organism's genetic makeup. Phenotypes range from physical to behavioral characteristics with variations resulting from mutation. Mutations reveal how genes function in controlling phenotypes and what happens when the gene machinery is flawed. Unique behavior resulting from mutation is studied in an assortment of organisms, including the fruit fly, *Drosophila melanogaster*.

***Drosophila melanogaster* as a genetic model**

The organism *Drosophila melanogaster* is a convenient genetic model with the ability to produce a substantial amount of progeny within a short period of time. The costs of acquiring and maintaining fly stocks are relatively less expensive than the acquisition and upkeep of other organisms. Another advantage of using *Drosophila* is the relative simplicity of its genetics. With only one pair of sex and three pairs of autosomal

This thesis follows the style of Developmental Dynamics.

chromosomes, it is easier to track mutations on loci of certain genes. Furthermore, a variety of techniques are utilized to analyze gene expression in the fly.

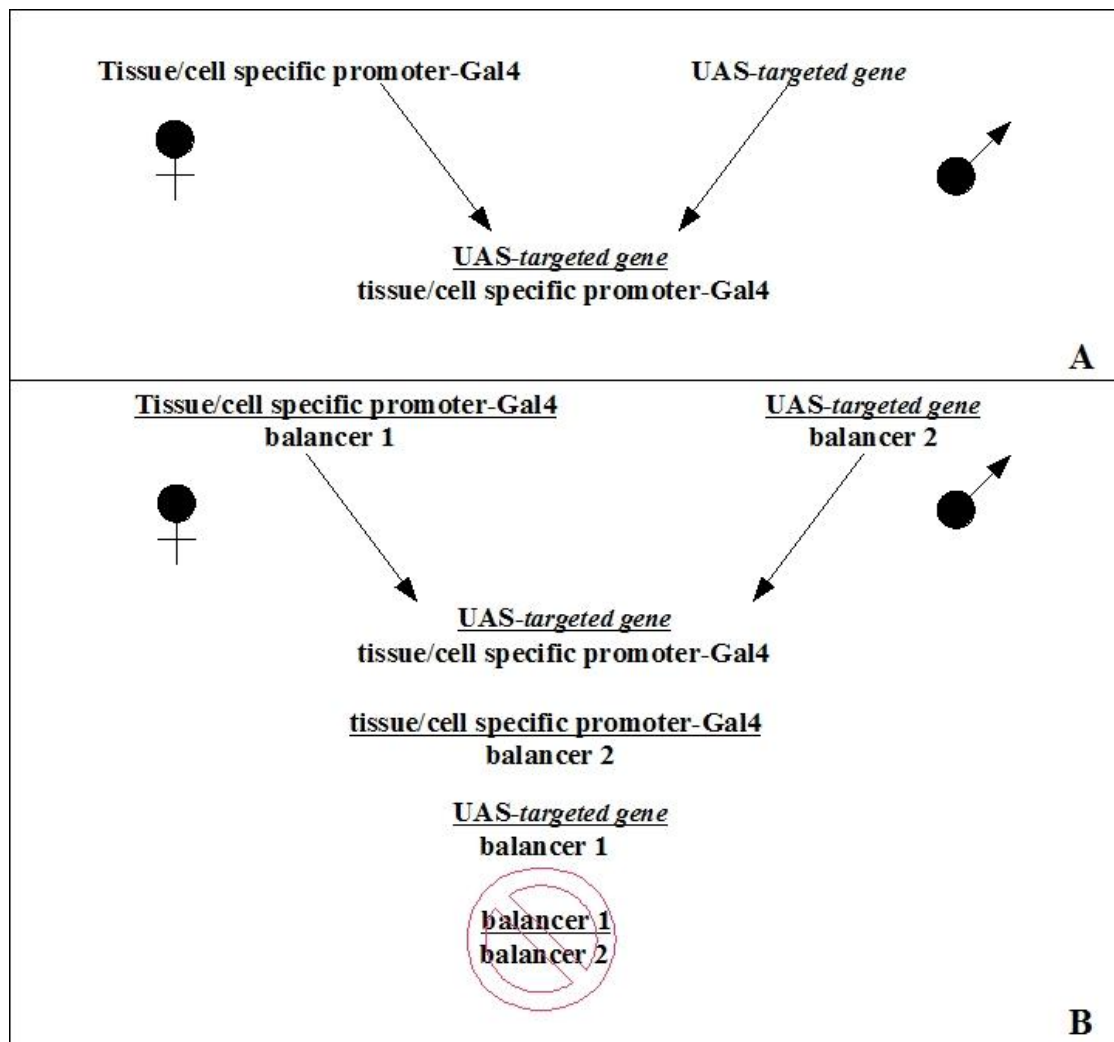


Fig. 1. Possible genotypes of progeny from homozygous and heterozygous parents with either the UAS reporter or Gal4 driver. **A:** Homozygous parents produce only one progeny genotype with both constructs. **B:** Heterozygous parents produce only three viable types of progeny. Flies without the reporter or driver (have only the balancers) are lethal, as represented by the cross-out symbol.

The UAS/Gal4 system is a common genetic tool used to drive expression of a gene in a specified set of cells (Brand and Perriman et al., 1993). This driver-reporter system consists of four components with two of the components belonging to either the driver or reporter. The driver consists of a tissue or cell-specific promoter and a Gal4 transcription factor. The reporter consists of an Upstream Activation Sequence (UAS) construct and the gene of interest. To utilize this system, each parent fly contains the driver or reporter line. These parents are mated to produce a percentage of the progeny with the genotype consisting of both components of the system (Fig. 1). The mechanism behind the UAS/Gal4 system has been identified (Brand and Perriman, 1993). In this system, the tissue or cell-specific promoter activates the transcription factor protein Gal4, which then binds to the UAS construct. The binding of Gal4 to UAS activates the expression of the gene of interest downstream from the UAS promoter (Fig. 2). Expression of fluorescent markers such as GFP or an RNA interference construct can be activated in a desired subset of cells. The system is used to over-express genes and reduce gene expression as well as to label expression through markers. Patterns of localization can provide information on genetic functioning and elicitation of certain behaviors.

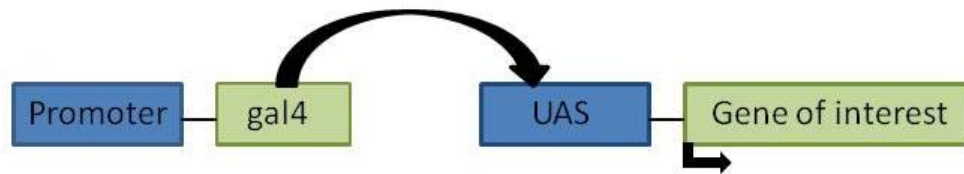


Fig. 2. The UAS/Gal4 system. A tissue or cell specific promoter activates the Gal4 transcription factor which will bind to the Upstream Activation Sequence (UAS). This will activate expression of the targeted gene. Additionally, RNA interference (RNAi) can knock-down expression of a particular gene through the UAS/Gal4 system.

Ovulation and oviposition

Female reproductive behavior can be segregated into two processes: ovulation and oviposition. During ovulation, eggs mature and shift from the ovaries to the uterus. Eggs first develop at the tip of the ovariole; maturation continues as the newly formed egg advances downwards. The mature egg is then released from the bottom of ovariole to the lateral oviduct (Middleton et al., 2006). The egg travels through either the right or left lateral oviduct and into the converging common oviduct and uterus. If copulation has occurred, the sperm stored in the spermathecae, two rounded organs on both sides of the common oviduct, will fertilize the egg in the uterus. The female will further lay the egg through a process called oviposition.

Individual nerves innervate muscles of the female reproductive tract in order to ease eggs from the ovary to the external substrate. These nerves are divided into two categories: AbNvOv and AbNvUt (Middleton et al., 2006). AbNvOv refers to the abdominal nerve to the ovary while AbNvUt refers to the abdominal nerve to the uterus (Fig. 3). These nerves are essential for egg movement (Middleton et al., 2006).

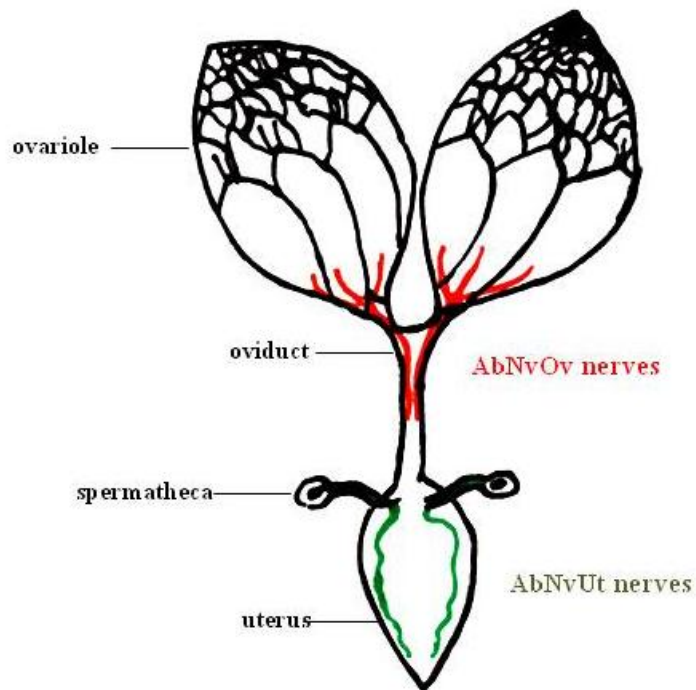


Fig. 3. Anatomy of the female reproductive tract. Ovulation starts at the tip of the ovariole and matures to the base of the ovary. The AbNvOv nerves aid the egg through the oviduct and into the uterus to be fertilized by sperm stored in the spermathecae. The AbNvUt nerves innervate the uterus in order for egg laying to occur (Middleton et al., 2006).

Female reproductive behavior is regulated by an assortment of genes, including a particular set known as the *p24* gene family.

The *p24* gene family

p24 genes are present in many organisms, ranging from yeast to flies to mammals.

These genes can be divided into four subfamilies: alpha, beta, gamma, and delta. A few organisms do not need all *p24*s for full functionality; for example, plants only contain the beta and delta subfamilies (Carney and Bowen, 2004). However, the organism *Drosophila melanogaster* has representation from all four subfamilies. Among the

Drosophila p24 subfamilies, there are nine *p24* gene loci that encode for transmembrane proteins around 24 kD (Boltz et al., 2007). These p24s are theorized to function in the trafficking of cargo vesicles between the endoplasmic reticulum (ER) and Golgi apparatus (Carney and Bowen, 2004). This occurs by the association with the COP I and COPII functions of the cell. COP II regulates vesicle movement from the ER to the Golgi while COP I regulates movement in the reverse manner from the Golgi to the ER (Boltz et al., 2007). Unfortunately, the precise functions of p24 proteins are still unclear. There have been hypotheses that p24s function in the production of these vesicles or the filtration of materials entering the vesicles (Carney and Bowen, 2004). When expression of a *p24* is reduced or lost, *Drosophila melanogaster* mutants are sterile. Specifically, when the expression of *p24* genes *logjam* (*loj*), *eclair* (*eca*), or *baiser* (*bai*) is lost, adult females do not lay eggs (Bartoszewski et al., 2004; Carney and Taylor, 2003). *Loj* protein has been shown to be expressed in the female central nervous system and the ovaries, localizing to the cytoplasm (Boltz et al., 2007). With evidence of where and how *Loj* works, we hypothesize other p24s will function similarly.

Role of neurotransmitters

Octopamine and glutamate are neurotransmitters necessary for muscle contractions of the ovarioles, oviducts, and uterus (Fig. 4).

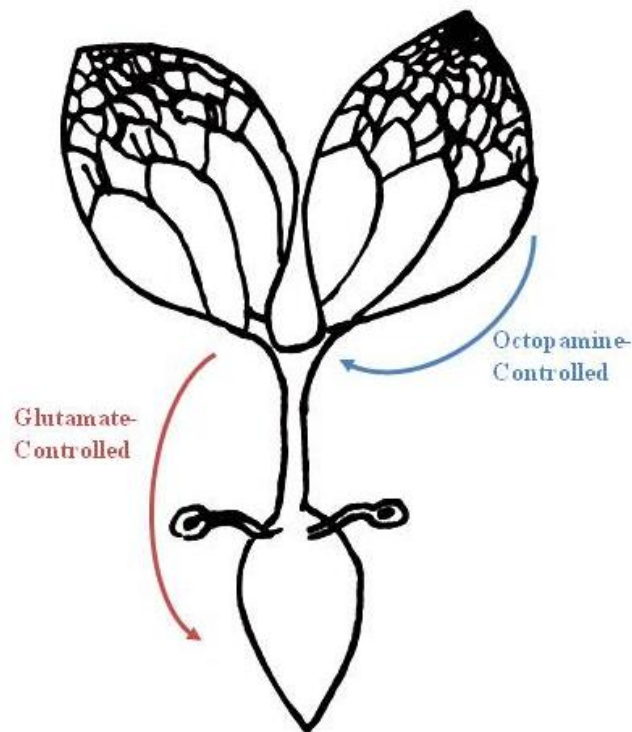


Fig. 4. Neurotransmitter control of the female reproductive tract. Octopamine relaxes the muscles of the oviduct and contracts the peritoneal sheath in order for the newly mature egg to travel to the oviduct. Glutamate then induces oviduct contractions so that the eggs pass down into the uterus (Middleton et al., 2006).

The full mechanism for uterine contraction has not yet been determined. However, there is research showing that octopamine and glutamate collectively work to contract and relax oviduct muscles (Rodriguez-Valentin et al., 2006). Octopamine relaxes oviduct muscles by inhibiting these muscular contractions and stimulating contractions of the peritoneal sheath surrounding the ovary, forcing the newly matured egg to shift to the oviduct (Middleton et al., 2006). Glutamate counteracts the effects of octopamine by inhibiting contractions of the sheath and inducing contractions of the oviduct to then shift the egg from the oviduct into the uterus (Rodriguez-Valentin et al., 2006). If the

animal discontinues production of octopamine or glutamate, eggs would not be able to pass through the oviduct and into the uterus to be oviposited. Thus, these females are considered sterile (Monasterioti et al., 1996; Cole et al., 2005). Studies have shown that the addition of one substance without the other would not be enough to fully rescue oviduct contraction and relaxation in vitro (Rodriguez-Valentin et al., 2006). However, it is possible that adding both substances could restore egg-laying in *p24* mutants.

Scope of the project

With *p24* gene function and location specificities unidentified, we propose that secretory proteins in cargo vesicles from the ER to the Golgi could possibly include egg-laying and other reproductive behavior signals. If trafficking of signals is blocked due to lack of *p24* gene function, then the neuropeptides controlling ovulation and oviposition possibly would not be secreted and thus eggs would not be oviposited. We conducted immunostainings with anti-p24 antisera in order to determine the localization of p24 proteins and specificities of p24 function. Preliminary immunostaining data confirmed that *Loj* co-localizes with markers for peptide-secreting cells, marking a relationship between p24s and peptide secretion. We plan to co-immunostain other p24s with peptidergic neuron markers to determine if there is an association between cells that express other p24s and those that control peptide secretion. Feeding experiments were conducted on female *loj* and *eca* mutants to determine if we could restore egg-laying by increasing the concentration of neurotransmitters that control egg movement. Our

analysis provides information contributing to the understanding of how genes control female reproductive behavior.

CHAPTER II

METHODS

Restoring egg laying in *Drosophila*

To restore egg laying in *Drosophila melanogaster*, we conducted feeding experiments with two neurotransmitters: octopamine and glutamate. The goal was to determine if the animals lack the ability to produce these chemicals. Animals were fed either glutamate and octopamine together or glutamate alone.

Components of the feeding solution

The yeast paste mixture contained blue food coloring, octopamine, glutamate, yeast, water, and the cornmeal mixture normally used for food.

Blue food coloring

To ensure that the chemicals had been consumed by the females, blue food coloring was added into the yeast paste. Females that ingested the chemical showed the dye in their gut tissues. Blue excrement was also seen on the sides of the glass vials. Four drops of blue dye per milliliter of water were added to a 15 mL tube. Altogether, 40 drops of dye were added to 10 milliliters of water.

Octopamine and glutamate

The chemicals, glutamic acid and octopamine hydrogen chloride, were obtained from Sigma Aldrich. Octopamine was tested with two final concentrations, 25 and 50 mg/mL, based on previous research (Cole et al., 2005 and Monasterioti et al., 1996). Since glutamate feeding experiments were not found in previous literature, final concentrations of glutamate started as close to 25 and 50 mg/mL as possible. The stock solution concentration (originating concentration) was 12.5 mg/mL, created by adding 1.25 grams of each chemical to 10 mL of purified water.

Yeast paste

One milliliter of the blue food coloring solution and 300 milligrams of inactivated yeast were added to a 1.5 mL tube. Two hundred microliters of each chemical were added to the yeast paste mixture. These tubes were capped and vortexed at the highest speed for one minute to ensure complete mixture of the components needed to make the paste. One hundred fifty microliters were taken from the yeast paste stock and pipetted in the center of the top of the cornmeal food. After swirling vials a couple of times to evenly spread the yeast paste, vials were refrigerated overnight to harden. One milliliter of the yeast paste solution provided seven vials of food. The treated food was then ready to be used at any time.

Feeding the fly

Octopamine and glutamate were mixed together into a yeast paste spread on top of the cornmeal within the vial. To consume cornmeal, the animals needed to consume the yeast paste. A yeast paste control was created with no substances added. Virgin female mutants (*eca cu sre/Df(3R) GB104; bai^{d09741}/bai cu sre*) and controls (*eca cu sre/TM3, Sb* and *Df(3R) GB104/TM3, Sb; bai^{d09741}/TM3, Sb* and *bai cu sre/TM6b, Tb*) were individually collected and aged for three to five days. Each female was grouped with two CS males that were three to five days old. For five days, flies were tossed into new vials and eggs were counted. The number of eggs laid by the experimental and control groups were compared. Females without blue dye in their gut tissues were excluded from the egg count.

Characterizing p24 protein expression

A method of determining functionality of a gene or gene family is to pinpoint the location of the gene product, or protein. This method of characterizing protein expression can be accomplished by immunostaining. Immunostaining is a two day process using two antibodies to bind and tag a particular protein expressed in a certain area of tissue. Each primary antibody is directed against a specific p24 protein; therefore we tested nine separate affinity purified antisera. The secondary antibody has a fluorescent tag that labels where the protein is located. The goal of p24 antibody immunostaining is for the primary antibody to bind to the cells where the p24 proteins are expressed and for the secondary antibody to fluoresce in particular patterns and

TABLE 1. Expected Expression of <i>p24</i> Transcripts						
	Non-sex specific tissues		Female tissues		Male tissues	
<i>p24</i>	CNS	Gut	Spermatheca	Ovaries	Accessory gland	Testis
alpha						
<i>eclair (eca)</i>	++	+++	++++	+++	++++	++
<i>p24-2</i>	+	+	+	+	++	-
beta						
<i>CHOp24</i>	++	++	++++	+++	++++	++
<i>CG9308</i>	-	-	-	-	-	+
delta						
<i>baiser (bai)</i>	++	+++	++	+++	++++	++
gamma						
<i>logjam</i>	++	+++	+++	+++	++++	+
<i>p24-1</i>	+	+	+	+	++++	+
<i>CG9053</i>	++	+++	++	+++	++	+
<i>CG31787*</i>	-	-	-	-	-	++++

Transcripts of the nine *p24* genes are expected to be expressed in particular tissues of *D. melanogaster* adults. Expression is characterized by the strength of the mRNA signal, ranging from ‘-’ (no signal) to ‘++++’ (strong signal). **CG31787* is male specifically expressed; therefore, expression in non sex-specific tissues (i.e., brain, gut) is limited to males only (Boltz et al., 2007). Data drawn from FlyAtlas (Chintapalli et al., 2007).

Concentrating the antisera

Two of the nine antisera were concentrated using iCON™ Concentrators obtained from Pierce. The original two milliliter antisera samples (for CHOp24 and CG9053 separately) were placed into the upper chamber of the concentrator. Using a fixed-angle rotor at 6000g and 22 degrees Celsius, samples were centrifuged at two minute intervals for ten minutes. The final volume for the CHOp24 antiserum was 380 microliters. For the CG9053 antiserum, the final volume was 400 microliters.

The process of immunostaining

Day one

Tissues were dissected in a 1X phosphate buffered saline (PBS) solution. These tissues were then fixed in a 4% paraformaldehyde (PFD) in 1X PBS solution for 20 minutes. The PFD solution was bound to the tissues to keep their structures fixed. This prevented tissue degradation during the immunostaining process. Multiple washes with PBS and PBST (PBS and 0.1% of the detergent Triton X) were completed to wash off the remaining PFD. The tissues were incubated in a solution of 10% goat serum in PBST for 30 minutes in order to block nonspecific sites in the tissue. This action prevented the antibody from binding to both nonspecific and specific sites. The primary anti-rabbit antibody (anti-p24) was then added to the tissues and goat serum solution and incubated overnight. The concentration of the antibody solution varied depending on which p24 antiserum was being tested. During this step, the primary antibody was bound to the specific p24 protein.

Day two

The primary antibody solution on the second day of the immunostaining process was removed and stored in the 40 degree Celsius refrigerator for future use. To remove unbound primary antibody, the tissues went through four washes with PBST: two long 30 minute sets followed by two quick rinses. The tissues were submerged in a solution of 10% goat serum and PBST to block the nonspecific sites of the protein. The secondary goat anti-rabbit antibody was then added, and the solution was incubated for one hour in tinfoil to prevent the fluorescence of the antibody from fading. After removal of the secondary antibody, tissues underwent an additional four washes with PBST: two 30 minute sets followed by two quick rinses similar to the first set of washes. Washes ensured the removal of unbound secondary antibody. Lastly, tissues were mounted on slides using Prolong Gold Antifade obtained from Invitrogen to prevent fluorescent markers on tissues from degrading. Slides were then viewed for staining.

Antibody specificity

To confirm p24 localization patterns, we must verify that each p24 antiserum is specific to its corresponding p24 protein. The best way to test specificity is to compare signal in a *p24*-deficient animal (mutant) to signal of its wild-type sibling. In our lab, we are able to create mutants for only three *p24* genes: *eca*, *bai*, and *loj*. Since *loj* had already been characterized (Boltz et al., 2007), we aimed to compare signal of *eca*- and *bai*-deficient animals to their respective wild-type siblings. To create mutants, *p24*-deficient animals were crossed into a deficiency background. The progeny of these animals included both

experimental (p24 with deficiency) and control (heterozygous p24 and deficiency) genotypes under the same background (Fig. 5).

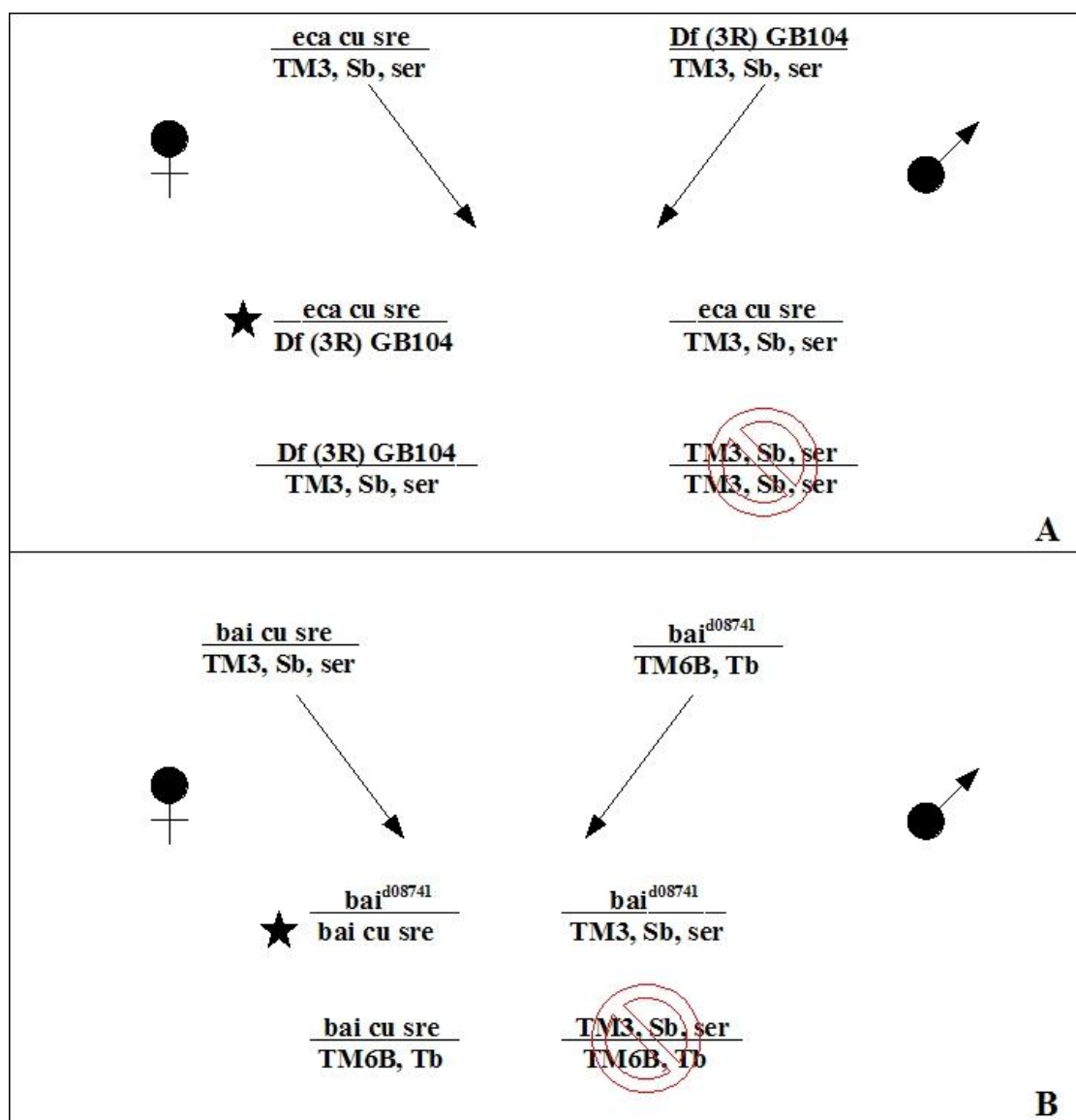


Figure 5. A-B: Possible genotypes for *eca* and *bai* mutant progeny. Animals with balancers over each other are lethal. The star symbol represents *p24* mutants and the cross-out symbol represents lethal animals.

Since we only have mutants for three of the nine *p24s*, we planned to test specificity for the remaining six antisera using transgenic constructs. Transgenic constructs are utilized to over-express a gene in an area where it is normally not expressed. To over-express a gene, experimental animals were produced using the reporter-driver system, UAS-GAL4. The lab created *p24* transgenics (UAS-*p24s*). We crossed transgenic lines with one of two Gal4 promoters: *SG18.1-gal4* or *CY2-gal4*. *SG18.1-gal4* is specific to neural tissues, such as the brain and ventral nerve cord, while *CY2-gal4* is specific to neural tissues and eggs. Depending on the driver used, the *p24* was expressed in a targeted tissue in which it is not normally expressed.

Mated transgenic females were aged three to seven days before dissection. With the experimental and control groups obtained, each underwent the immunostaining process as described for normally determining p24 protein expression.

CHAPTER III

RESULTS

Feeding assays

Testing yeast paste without chemicals

In order to determine if the animals would consume the yeast paste mixture, we conducted a food control experiment. Wild-type *CS* female flies were tested with chemically-untreated yeast paste. The wild-type flies were able to eat the paste, determined by the blue food coloring seen in their gut tissue and blue excrement. Eggs were observed clearly through the blue food coloring at the bottom of the vial. We later tested *eca* mutants and siblings with the same chemically-untreated yeast paste mixture. All animals tested consumed the food as displayed by the blue gut and the blue excrement on the walls.

Octopamine and glutamate pilot test

eca mutants and siblings were then fed a yeast paste mixture containing octopamine and glutamate. Our data showed that the concentrations of glutamate and octopamine used (25 mg/mL each) did not have an effect on egg laying when comparing the mutants to the sibling controls. The mutant experimental animals did not lay any eggs.

In order to alleviate the problem, the concentrations of octopamine and glutamate were increased in order to flood neuronal synapses with these neurotransmitters. The amounts

of glutamate and octopamine doubled to 50 mg/mL. We also tested an increased concentration of glutamate (50 mg/mL) with the original concentration of octopamine (25 mg/mL). Furthermore, we treated a separate set of food with only glutamate and tested animals with concentrations of 25, 50, and 100 mg/mL. With all these concentrations, none of the experimental animals displayed an increase in egg laying.

p24 characterizations

TABLE 2. Tested Expression of p24 Proteins								
	Female tissues					Male tissues		
p24	Brain	Fat	Eggs	Oviduct	Spermatheca	Accessory gland	Ejaculatory duct	Testis
Bai	+	+	-	-	+	+	ND	+
Eca	+	+	+	+	+	+	+	?
p24-1	+	+	+	+	+	+	+	+
p24-2	+	?	+	+	+	ND	ND	ND
CG9308	-	+	-	+	+	+	+	+

Signal of five p24 proteins in female and male tissues is analyzed using specific p24 antisera. The symbols ‘+’ and ‘-’ indicate signal or no detectable signal, respectively. Possible staining is represented with a ‘?’, and tissues not yet assayed are represented by ‘ND’. Localization patterns differ among p24s; for example, Baiser is localized to the optic lobe while p24-1 is ubiquitously expressed throughout the brain.

Eight of the nine p24 proteins were characterized according to immunostaining localization patterns (Table 2). Most expression data was gathered from female tissues. Tissues were obtained from three to seven day old mated female and male *CS* animals. Expression of each p24 was cytoplasmic. All p24 antisera were affinity purified before being tested on wild-type tissues. Each p24 immunostaining varied in pattern, localization, and intensity.

CG31787

Since *CG31787* is only expressed in males, female animals were not tested. We are in the process of testing *CG31787* expression and specificity.

Bai

The clearest immunostaining results occurred at two concentrations of primary antibody, 1:3 and 1:5, and two concentrations of secondary antibody, 1:1500 and 1:2000. Results indicated that Bai is localized to the brain, ventral nerve cord, fat, gut, and spermatheca of the female fly. Within the brain, cells were primarily stained in the optic lobe region. To test specificity, *bai* mutants were created by using a *bai* P-element insertion with a *bai* EMS deletion allele (Fig. 5B). Only a few *bai* mutants were collected due to the difficulty for the animals to live past the developmental stages. Immunostainings from sibling controls produced similar results to the wild-type *CS* immunostainings with signal in the optic lobe of brains, ventral nerve cord, spermatheca, and fat (Fig. 6). The mutant immunostainings did not show expression of Bai in any tissue (Fig. 6).

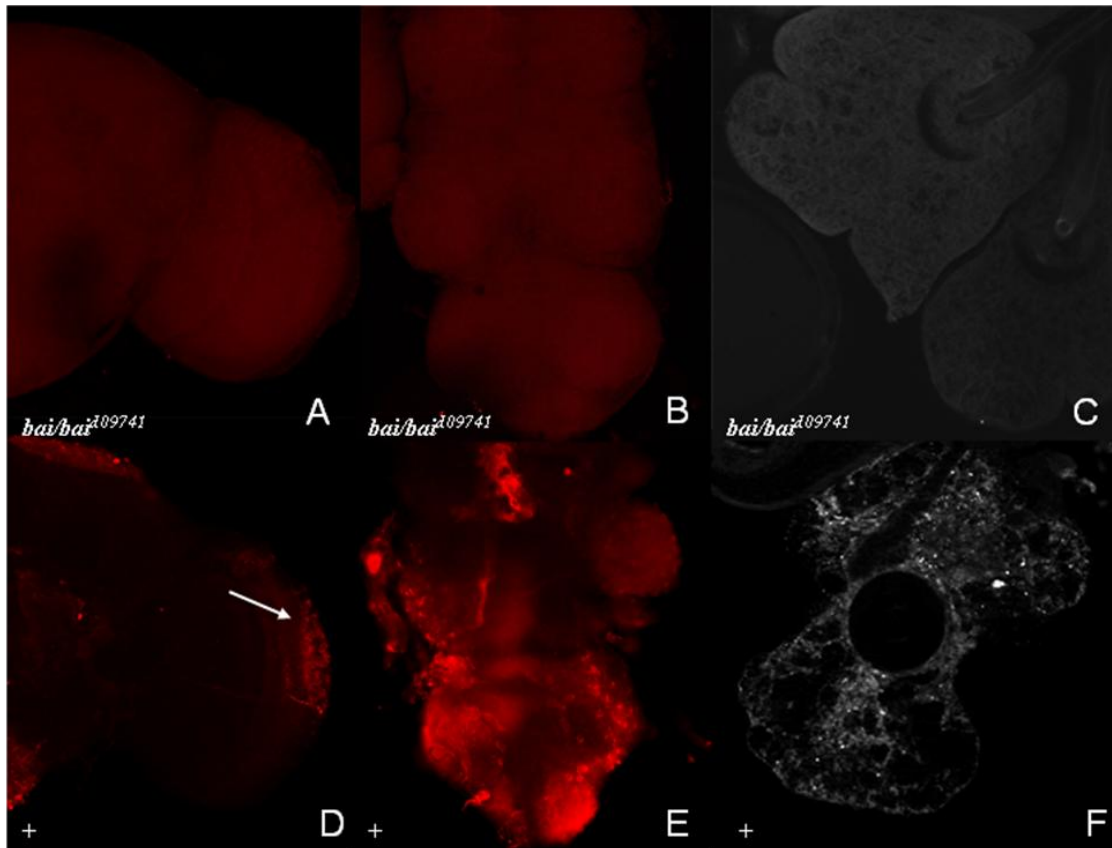


Fig. 6. Anti-Bai antiserum confirms specificity to the Bai protein. A-C: Expression of Bai in *bai* mutant tissues. D-F: Expression of Bai in the CS wild-type control. Arrow in (D) points to signal in the optic lobe of the brain. Tissues photographed include the optic lobe of the brain (A, D), ventral nerve cord (B, E), and spermatheca (C, F). Photos at 20X.

Eca

Optimal expression occurred with the following concentrations- 1:300 and 1:50 for the primary antibody and 1:1500 and 1:2000 for the secondary antibody. Eca is expressed in the brain, ventral nerve cord, fat, eggs, oviduct, accessory gland, and spermatheca (Fig. 7). Staining was observed throughout the central portion of the brain in large cells that are likely to be neurosecretory.

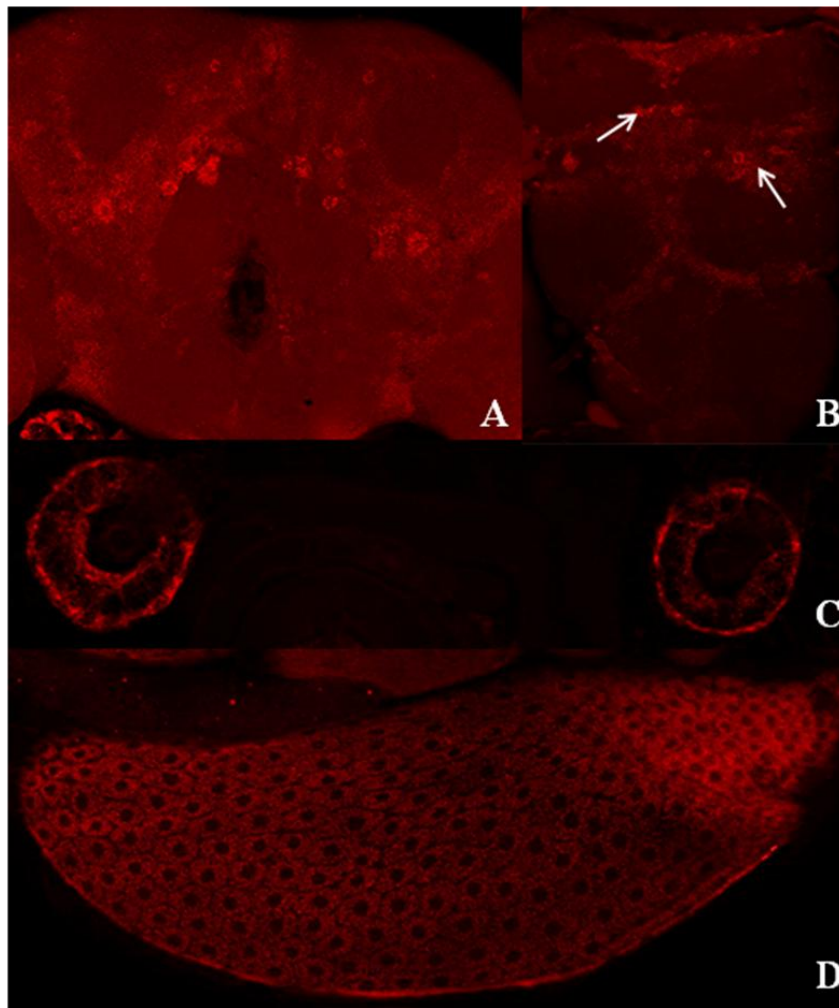


Fig. 7. Wild-type Eca localization in the central nervous system, spermatheca, and eggs. **A:** Eca is ubiquitously expressed in the brain. **B:** Eca is expressed in certain areas of the ventral nerve cord (arrows). **C:** Signal is seen outlining the spermathecae. **D:** Punctate dots of an anti-Eca stained egg are observed. Photos taken at 20X.

Specificity of the Eca antiserum

eca P-element insertions were placed into a deficiency background to produce mutants and wild-type siblings (Fig. 5A). Eca expression in the sibling controls was similar to Eca expression in the *CS* wild-type. The mutant did not show any expression of *eca* in any of the tissues examined (Fig. 8)

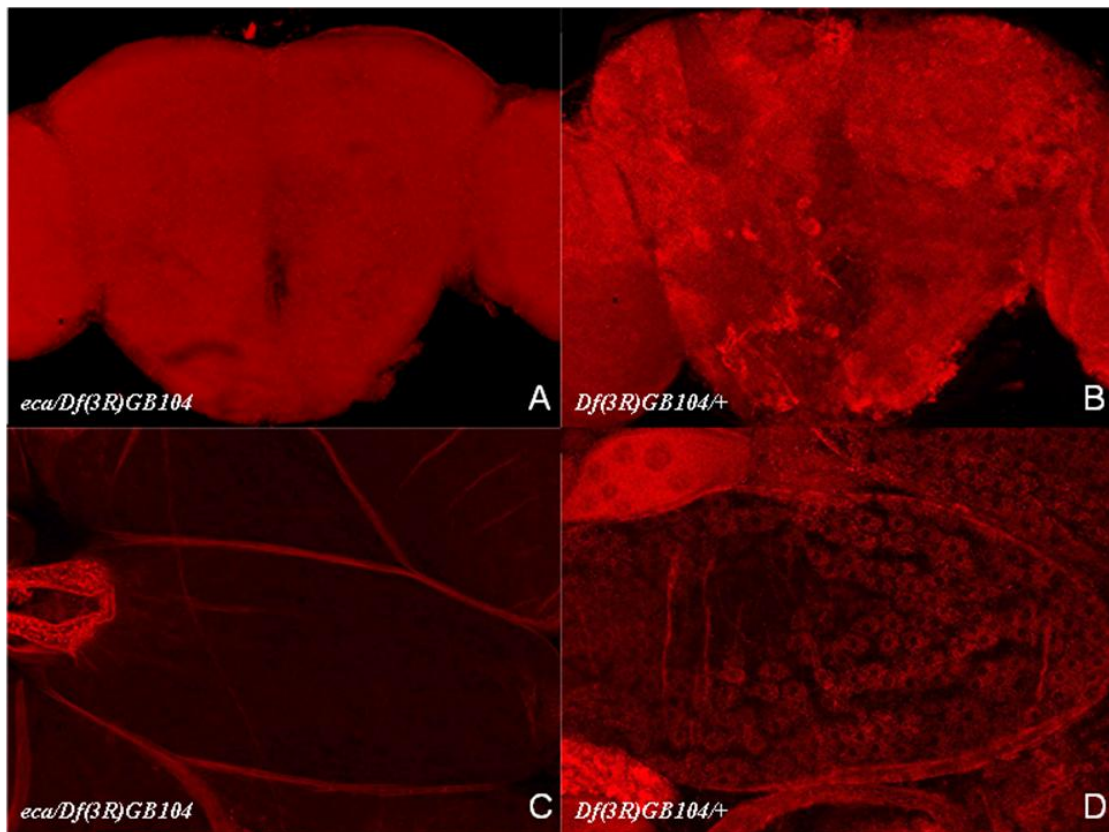


Fig. 8. Anti-Eca antiserum demonstrates specificity to the Eca protein. **A, C:** Staining in the brain (A) and eggs (C) of *eca* mutants. **B, D:** Staining in brains (B) and eggs (D) of the sibling controls. Photos taken at 20X.

CG9308

The best concentrations to observe staining for this antiserum were 1:10 for the primary and 1:1500 for the secondary antibody. CG9308 is expressed in the following tissues: ventral nerve cord, fat, oviduct, accessory glands, and spermatheca (Fig. 9). Four cells (two on each side) in the lower abdominal ganglion were distinctly stained (Fig.9 C, D).

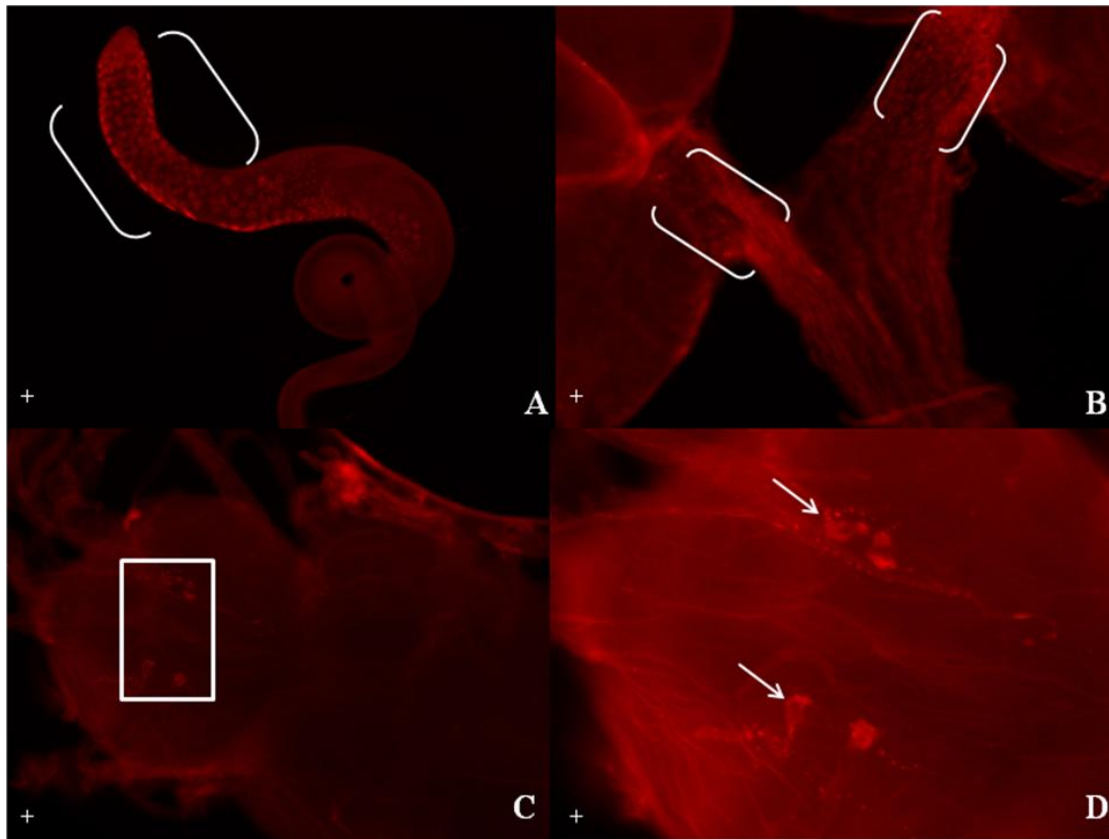


Fig. 9. Wild-type expression of CG9308. **A-B:** CG9308 is expressed in a variety of tissues, including the male testis (A) and female oviduct (B). **C-D:** CG9308 signal in the ventral nerve cord. (D) illustrates a magnified view of the four stained cells in the abdominal ganglion of the nerve cord. Photo taken at 10X for (A) and (C) and at 20X for (B) and (D).

Specificity of the CG9308 antiserum

Without a *CG9308* mutant, the best way to test the antiserum for specificity was to use an overexpression experiment. Since we did not observe staining in the brain or eggs, *CG9308* was over-expressed in these tissues. Over-expression was observed in the brain, nerve cord, and eggs with the *UAS-9308^{c107d}* strain. CG9308 was expressed throughout the brain as bright uniform speckles and in the ventral nerve cord as the four

cells previously seen in *CS* tissues. CG9308 signal was observed in mid-stage eggs rather than eggs already fully matured (Fig. 10).

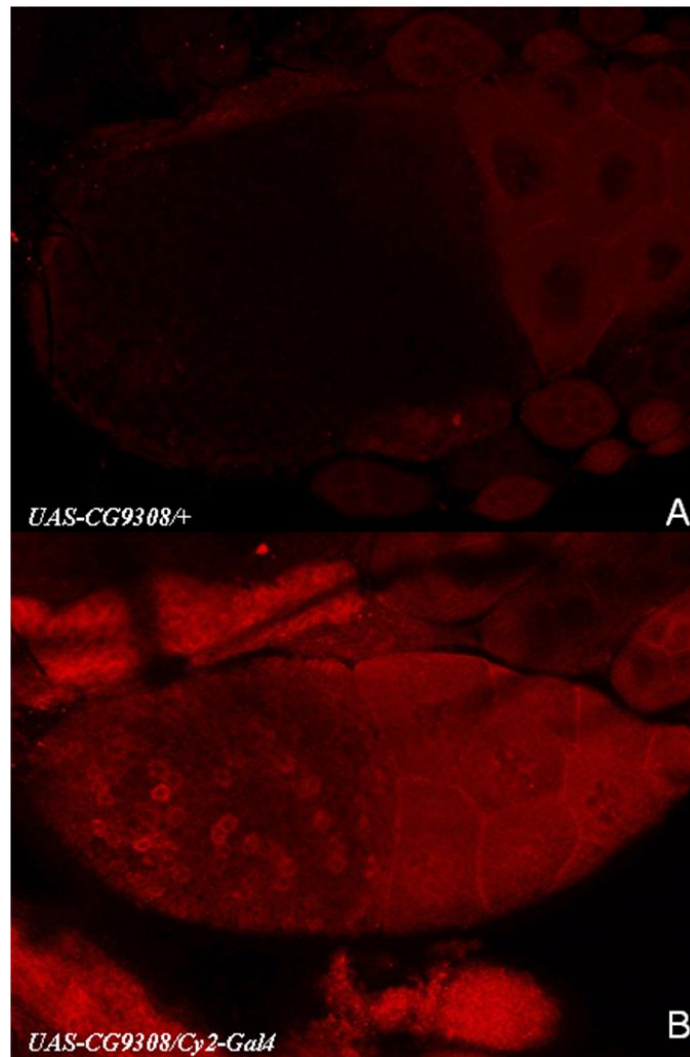


Fig. 10. Anti-9308 antiserum binds to the CG9308 protein. **A:** CG9308 is not normally expressed in eggs. **B:** CG9308 expression can be detected when it is over-expressed. Photos taken at 20X.

p24-1 (previously known as CG1967)

p24-1 signal is present in all tissues of the fly (Figs. 11-14). Signal in each tissue is distinct; punctate dots are stained in the cytoplasm of cells within the eggs (Fig. 13).

p24-1 is ubiquitously expressed in the brain and nerve cord (Fig. 11). There is also a unique pattern of cells stained in the seminal receptacle (arrows in Fig. 12E). Not only is p24-1 expression present in female tissues, it is present in male tissues as well (Fig. 14).

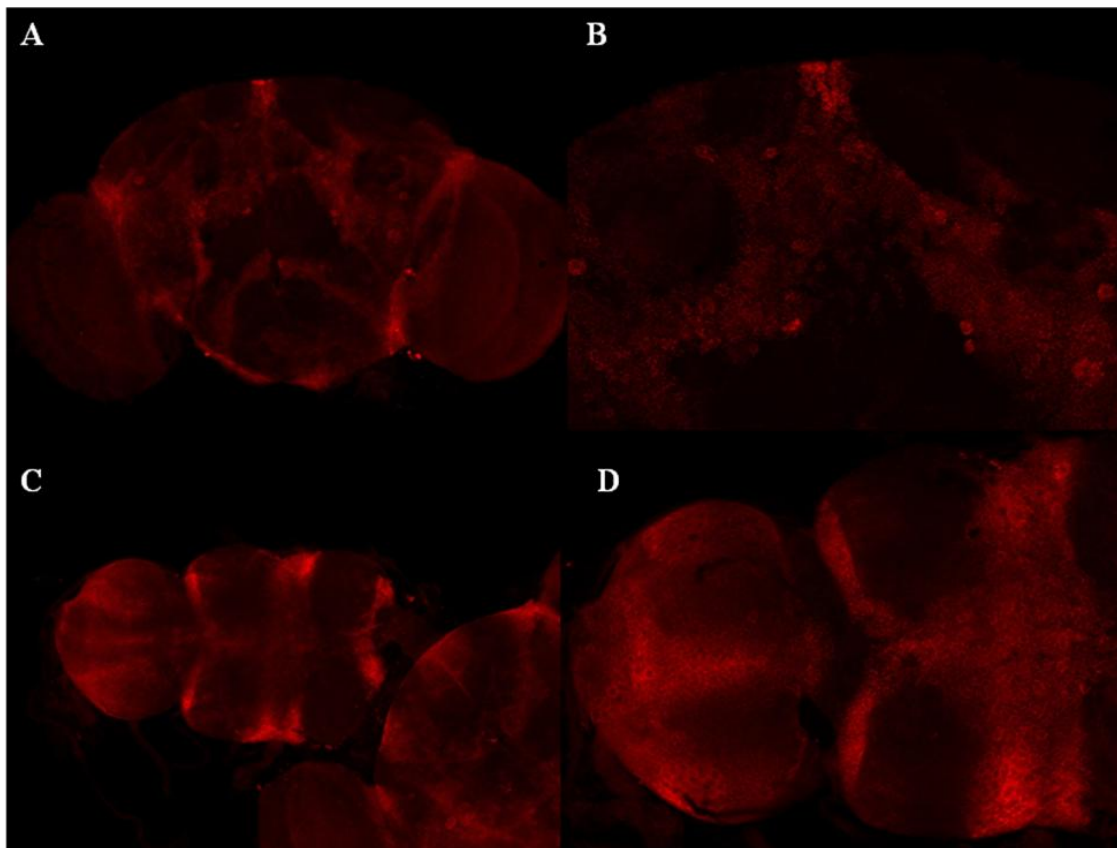


Fig. 11. Wild-type p24-1 expression in the central nervous system. (B) and (D) represent magnified views of (A) and (C) respectively. **A-B:** Expression is localized to the brain. **C-D:** Expression is localized to the ventral nerve cord. Photos were taken at 10X for (A) and (C) and 20X for (B) and (D).

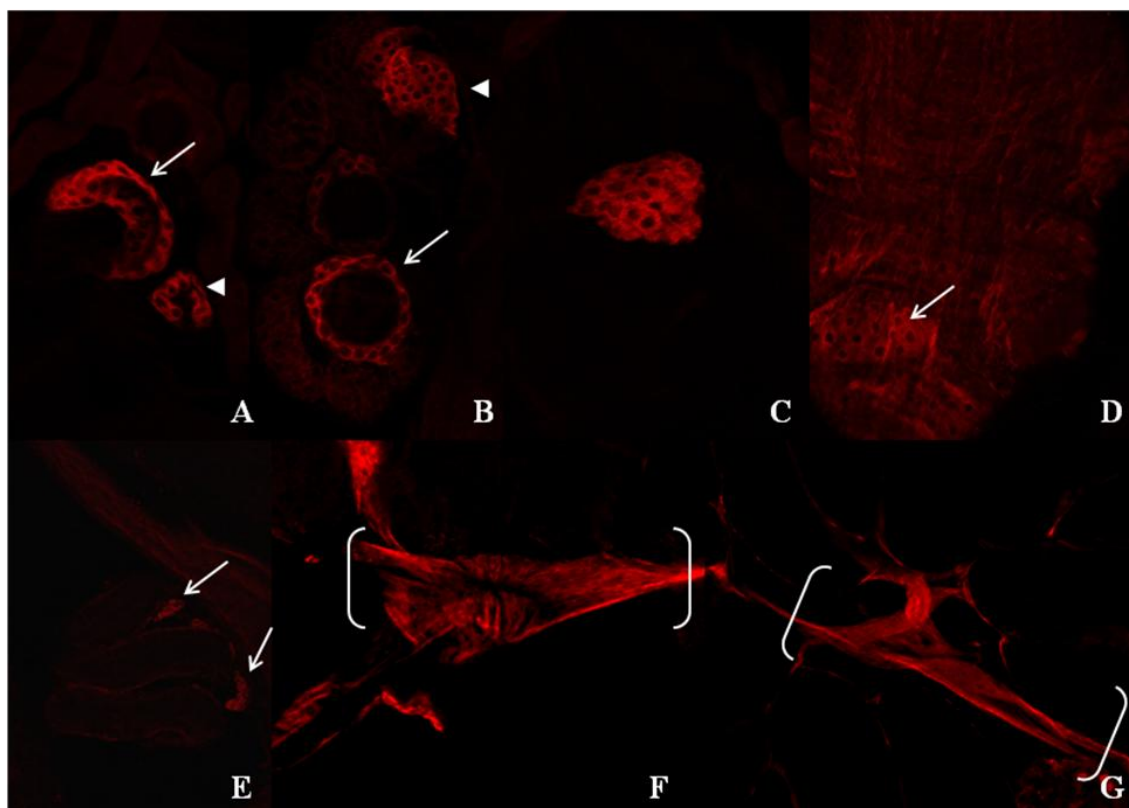


Fig. 12. p24-1 is expressed in various tissues of the female reproductive tract. **A-B:** Stainings of spermathecae (arrows) and accessory glands (arrowheads). **C:** Magnified view of staining in the accessory gland. **D:** Staining in the rectum. **E:** Signal observed on the edges of the seminal receptacle. **F-G:** Stainings of the common and lateral oviducts. Photos taken at 10X (G) and 20X (A-F).

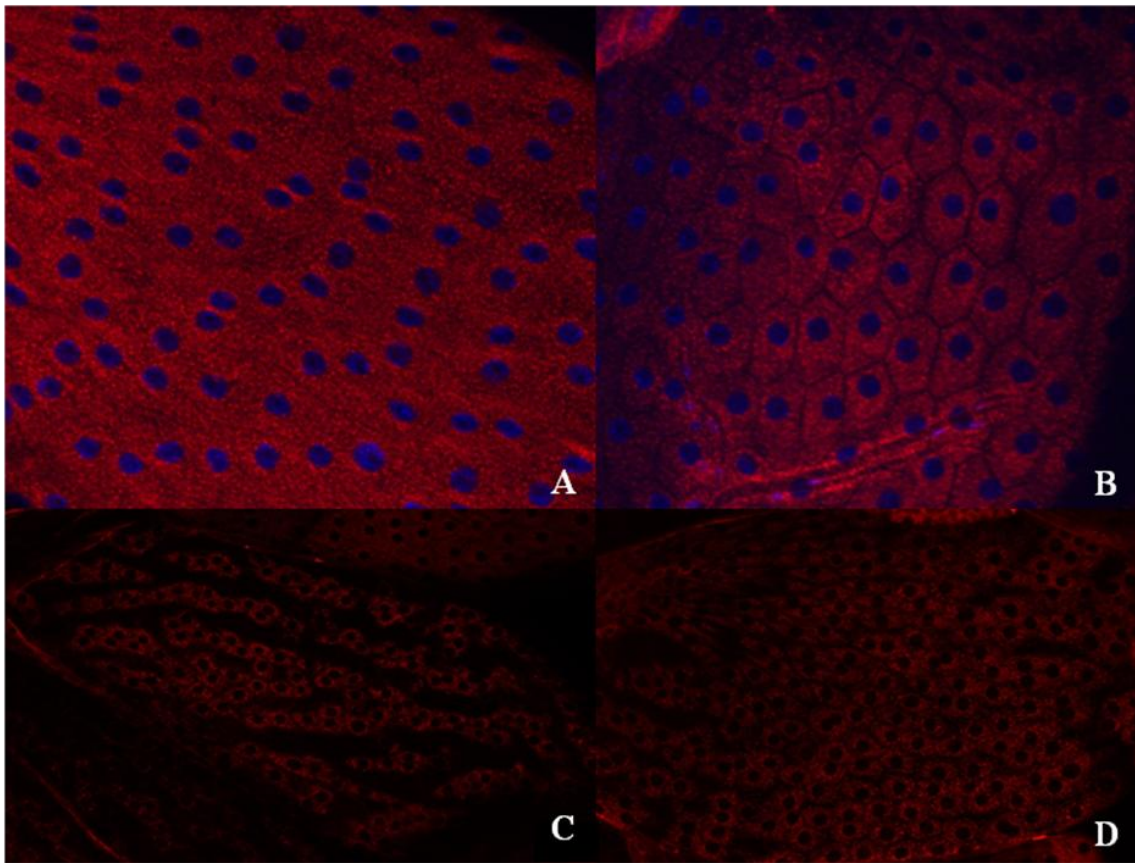


Fig. 13. p24-1 expression in eggs. **A-B:** Immunostainings of anti-p24-1 with the Hoescht dye, a nuclear marker, in two eggs. Punctate staining observed in these cells. **C-D:** p24-1 staining in two mature eggs. Photos taken at 20X in (C) and (D) and 40X in (A) and (B).

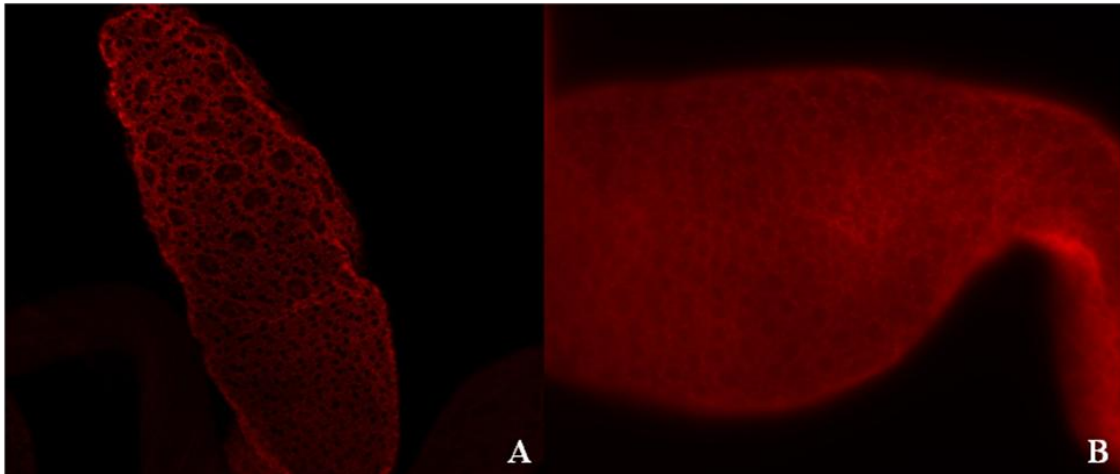


Fig. 14. Expression of p24-1 in wild-type males. **A:** Signal is seen in the cytoplasm of cells within the male accessory gland. **B:** Signal is seen in punctate dots throughout the cytoplasm of cells in the male seminal vesicle. Photos taken at 20X (A) and 40X (B).

Specificity of the p24-1 antiserum

Since no mutant was available and over-expression experiments could not be conducted due to expression in all tissues, we are exploring other methods of testing specificity.

p24-2 (previously known as CG33105)

The concentration of primary antibody used was 1:400 with a 1:1500 secondary concentration. The brain, ventral nerve cord, eggs, accessory glands, and spermatheca all showed expression of p24-2. Staining was ubiquitous in the brain and nerve cord. Signal was only observed as punctated dots in younger eggs. The spermatheca staining was very distinct with bright dots outlining the outside layer (Fig. 15).

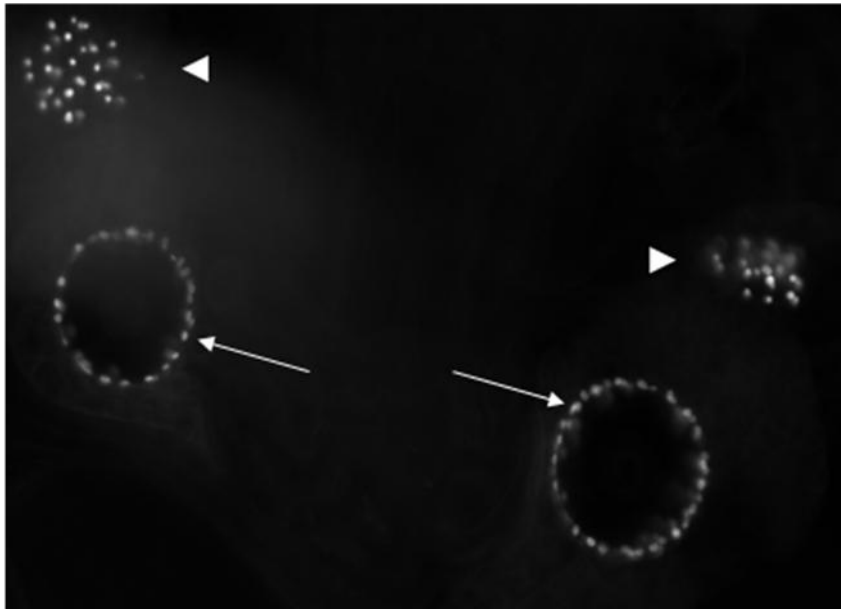


Fig. 15. p24-2 spermathecae expression. The spermathecae (arrows) and female accessory glands (arrowheads) show a unique pattern of p24-2 expression.

Specificity of the p24-2 antiserum

Specificity of this antiserum has not been tested due to lack of a specificity method in the laboratory at the moment. We are exploring other methods to confirm real signal.

CHOp24 (previously known as CG3564)

After concentrating the antiserum four-fold, the optimal concentrations for staining were 1:10 for the primary and 1:1500 for the secondary. CHOp24 was determined to be expressed in the brain, fat, and gut. The eggs did not stain; however, the sheath surrounding the eggs stained. Bright speckles were observed throughout the eggs and brain, yet it could not be determined if the staining was real or background. Potential

signal was localized to cells in the optic lobes and the P1 region of the central part of the brain. We are still in the process of characterizing expression in these tissues.

Specificity of the CHOp24 antiserum

No mutants were available to test the CHOp24 antiserum. However, over-expression experiments were conducted. Two *CHOp24* strains were tested (*UAS-CG3564^{L108b}/CyO* and *UAS-CG3564^{L120a}*) with one driver, *CY2-gal4*. Unfortunately, the antiserum was too weak to observe any staining. We are in the process of determining if another specificity experiment should be conducted.

CG9053

After not observing any staining with the primary antibody at a 1:1 concentration, the antiserum was concentrated to a four-fold increase. Results showed that the antiserum was still too weak to view any real staining. There was possible staining localized to the accessory glands, seminal receptacle, gut, and spermatheca.

Specificity of the CG9053 antiserum

No mutants were available for *CG9053*. Instead, we used the antiserum to detect over-expression in the fly. Two *CG9053* strains were tested (*UAS-CG9053^{G132a}/CyO* and *UAS-CG9053^{G102b}/CyO*) with one driver, *CY2-gal4*. After testing multiple concentrations of primary and secondary antibodies, it was determined that the antiserum was too weak to see any staining. We are in the process of determining if another specificity method should be used.

Expression of Cy2-Gal4

Regarding the results of the CHOp24 and CG9053 over-expression data, we were concerned that the driver used was not functioning properly. Therefore, we drove GFP expression in Cy2-Gal4 cells in order to confirm the Cy2 pattern and functionality (Fig. 16). We observed the normal Cy2 signal in the ovaries, nerve cord, and brain, confirming driver expression.

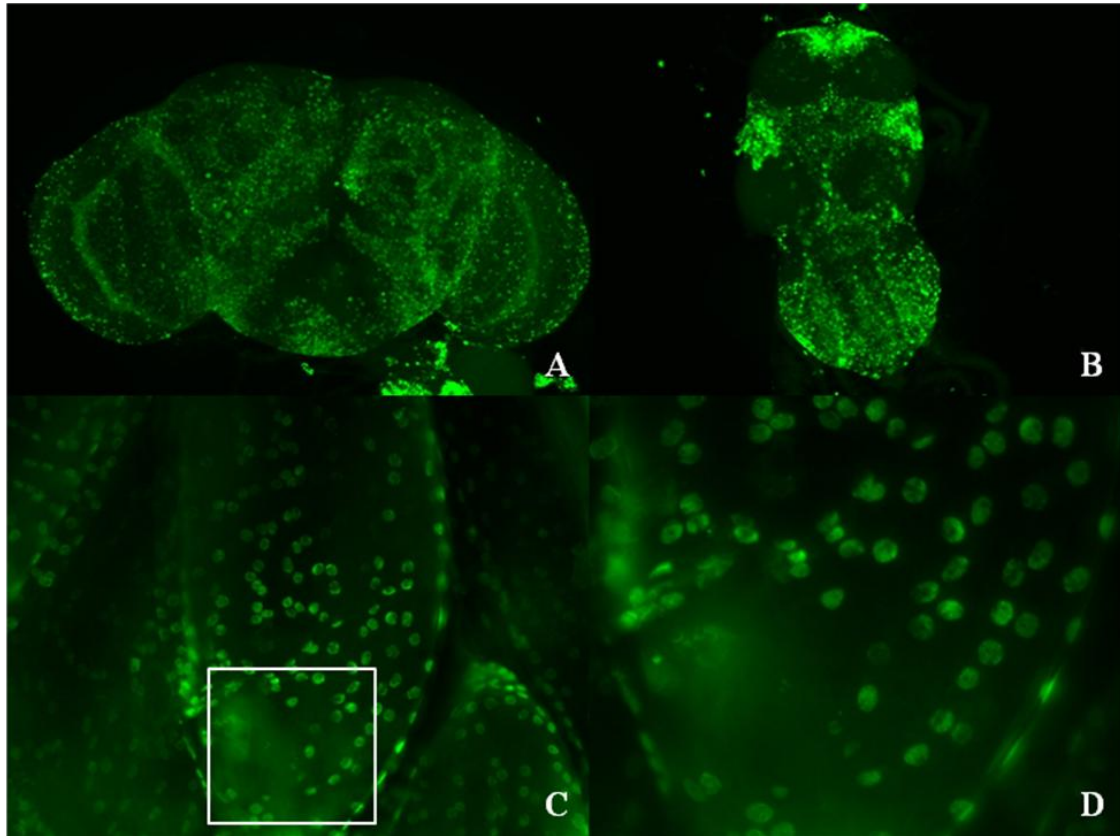


Fig. 16. CY2 driver staining in the central nervous system and eggs. **A-B:** CY2 is expressed throughout the brain (A) and nerve cord (B). **C-D:** CY2 is expressed within large cells in eggs. (D) is a magnified representation of boxed region in (C). Photos (A)-(C) taken at 20X; (D) taken at 40X.

CHAPTER IV

CONCLUSIONS

Feeding assays

After testing multiple concentrations of glutamate and octopamine, we determined that feeding the *eca*-deficient animals these chemicals does not restore egg laying. There are a variety of possible explanations for these results. The most likely explanation is that the animals do produce the necessary amounts of octopamine and glutamate; however, there may be a problem with the receptors for these chemicals. With this problem, there will be no reuptake or uptake of these neurotransmitters. A future experiment to conduct would be to compare localization patterns of wild-type octopamine/glutamate receptors with receptors of *eca*-deficient animals through fluorescent immunostaining. It also may be possible that there could be another chemical needed to induce egg laying. Studies on proctolin indicate it regulates egg-laying behavior in honey bees and grasshoppers (Miranda et al., 2003; Lange et al., 1987). Conducting feeding assays with proctolin may provide more information.

p24 characterizations

Our comparison of p24 expression illustrates that many are unique in pattern and area of localization (Table 2, Figs. 6-15). Many p24s are highly expressed in the ovaries, spermathecae, and central nervous system. We are in the process of classifying other

p24 expression patterns in specific tissues. p24 localization patterns will give us a better understanding of the processes regulated by these genes.

To confirm specificity of Eca and Bai antisera to their corresponding p24 proteins, we compared immunostainings of mutants to the wild-type siblings; we were able to see a difference in signal (Fig. 6, 8). When we over-expressed *CG9308* in tissues normally not showing expression, we detected anti-*CG9308* immunostaining (Fig. 10). Overall our data confirmed that the antisera bind specifically to Eca, Bai, and *CG9308*. We are still conducting specificity experiments on other p24s using over-expression constructs. Unfortunately both the *CHOp24* and *CG9053* antisera do not bind well to the protein and are too weak to observe any signal using over-expression. After testing functionality of the *Cy2-gal4* driver, we confirmed that the driver is working (Fig. 16). There may be a problem with both UAS constructs; however, it seems more likely that the antisera are weak since signal is not clear in wild-type *CS* flies.

A specificity method we are considering is usage of p24-RNA interference (p24 RNAi). RNAi is used to knock down expression of a gene of interest, particularly a *p24*. By combining a *UAS-p24 RNAi* construct with an *actin-Gal4* driver, animals with reduced p24 expression and wild-type siblings are available for immunostaining. However, our lab has shown that these animals with reduced p24 expression do not survive development. Therefore, to combat this issue, we would need to use a *Gal80^{ts}* driver in addition to the *Gal4* driver. *Gal80^{ts}* is a temperature-sensitive driver similar to *Gal4*. At

22 degree Celsius, Gal80^{ts} competitively binds to Gal4, preventing Gal4 from binding to the UAS construct; therefore, the RNAi of interest is not expressed (Pilauri et al., 2005). Once animals eclose, they are transferred to a 29 degree Celsius incubator. At warmer temperatures, Gal80^{ts} disassociates from Gal4, so that Gal4 will then bind to the UAS construct and will activate the RNAi. We hope to see a difference in expression between immunostainings of the experimental *p24*-deficient animals and wild-type sibling controls. We will test this technique in the future.

After all p24 localization patterns have been identified and specificity has been determined, we plan to co-immunostain other p24s (besides Loj) with peptidergic neuronal markers. Once we determine expression patterns of these genes and co-localization relationships with peptide secretion, we can further explore *p24* function in the fly.

REFERENCES

- Bartoszewski S, Luschnig S, Dejeux I, Grosshans J, Nüsslein-Volhard C. 2004. *Drosophila* p24 homologues *eclair* and *baiser* are necessary for the activity of the maternally expressed Tkv receptor during early embryogenesis. *Mech Dev* 121: 1259-1273.
- Boltz KA, Ellis LL, Carney GE. 2007. *Drosophila melanogaster* p24 genes have developmental, tissue-specific, and sex-specific expression patterns and functions. *Dev Dyn* 236: 544-555.
- Carney GE, Bowen NJ. 2004. p24 proteins, intracellular trafficking, and behavior: *Drosophila melanogaster* provides insights and opportunities. *Biol Cell* 96: 271-278.
- Carney GE, Taylor BJ. 2003. *logjam* encodes a predicted EMP24/GP25 protein that is required for *Drosophila* oviposition behavior. *Genetics* 164: 173-186.
- Chintapalli VR, Wang J, Dow JAT. 2007. Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat Genet* 39: 715-720.
- Cole SH, Carney GE, McClung CA, Willard SS, Taylor BJ, Hirsh J. 2005. Two functional but noncomplementing *Drosophila* tyrosine decarboxylase genes. *J Biol Chem* 280: 14948-14955.
- Lange AB, Orchard I, Lam W. 1987. Mode of action of proctolin on locust visceral muscle. *Arch Insect Biochem* 5: 285-295.
- Middleton CA, Nongthomba U, Parry K, Sweeney ST, Sparrow JC, Elliott CJ. 2006. Neuromuscular organization and aminergic modulation of contractions in the *Drosophila* ovary. *BMC Biol* 4:1-14.
- Miranda CRE, Bitondi MMG, Simões ZLP. 2003. Effect of proctolin on the egg-laying activity of *Apis mellifera* queens. *J Apicult Res* 42: 35-38.
- Monasterioti M, Linn CE Jr, White K. 1996. Characterization of *Drosophila Tyramine Beta-Hydroxylase* gene and isolation of mutant flies lacking octopamine. *J Neurosci* 16: 3900-3911.
- Pilauri V, Bewley M, Diep C, Hopper J. 2005. Gal80 dimerization and the yeast *GAL* switch. *Genetics* 169: 1903-1914.

Rodriguez-Valentin R, Lopez-Gonzalez I, Jorquera R, Labarca P, Zurita M, Reynaud E. 2006. Oviduct contraction in *Drosophila* is modulated by a neural network that is both, octopaminergic and glutamatergic. J Cell Physiol 209: 183-198.

CONTACT INFORMATION

Name: Stephanie Theresa Grady

Professional Address: c/o Dr. Ginger Carney
Department of Biology
MS 3258
Texas A&M University
College Station, TX 77843

Email Address: stephanie_grady@hotmail.com

Education: B.S., Biology, Texas A&M University, May 2010
Undergraduate Research Scholar